Surface Morphology Differences in *Clostridium difficile* Spores, Based on Different Strains and Methods of Purification

Dmitry Malyshev[1*], Les Baillie[1]

[1] School of Pharmacy and Pharmaceutical Sciences, Cardiff University, Cardiff, United Kingdom

***Correspondence**

Dmitry Malyshev

malyshevd@cardiff.ac.uk

Keywords: spores, anaerobes, SEM, clostridium, hydrophobicity, exosporium

Abstract

Infections linked to *Clostridium difficile* are a significant cause of suffering. In hospitals, the organism is primarily acquired through the faecal-oral route as spores excreted by infected patients contaminate the healthcare environment. We previously reported that members of the *C. difficile* group varied widely in their ability to adhere to stainless steel and proposed that these differences were a consequence of variations in spore architecture. In this study of clinical isolates and spore coat protein mutants of *C. difficile* we identified three distinct spore surfaces morphotypes; smooth, bag-like and "pineapple-like" using scanning electron microscopy (SEM). The frequency of each morphotype in a spore population derived from a single isolate varied depending on the host strain and the method used to produce and purify the spores. Our results suggest that the inclusion of a sonication step in the purification process had a marked effect on spore structure. In an attempt to link differences in spore appearance with key structural spore proteins we compared the morphology of spores of CD630 to those produced by CD630 variants lacking either CotE or BclA. While SEM images revealed no obvious structural differences between CD630 and its mutants we did observe significant differences ($p<0.001$) in relative hydrophobicity suggesting that modifications had occurred but not at a level to be detectable by SEM.

In conclusion, we observed significant variation in the spore morphology of clinical isolates of *C. difficile* due in part to the methods used to produce them. Sonication in particular can markedly change spore appearance and properties. The results of this study highlight the importance of adopting "standard" methods when attempting to compare results between studies and to understand the significance of their differences.

1. Introduction

Clostridium difficile (recently renamed as *Clostridioides difficile)* is a Gram-positive anaerobic spore-forming bacterium and is currently the most common cause of antibiotic-associated nosocomial infection the US and UK [1, 2]. It is estimated that in 2015 the pathogen was responsible for 500,000 cases and 15,000 deaths in the US, according to the CDC [3]. The most recent data from the UK reported 12,798 confirmed cases in England in 2016-2017 [4]. While the incidence in the UK has reduced significantly from a peak of 60,000 cases in 2007, it still remains a significant cause of suffering. The additional costs associated with treating infected individuals, estimated to be between £4,000 ($5,500) and £8,000 ($11,000) impose a significant financial burden on healthcare systems [5]. Alterations in colonic microbiota, usually due to broad-spectrum antibiotic treatment, increase sensitivity to *C. difficile* infection and enable the vegetative organism to produce cytotoxins that destroy host intestinal epithelial cells [6].

In the hospital environment the organism is primarily acquired through the faecal-oral route contaminating the surrounding healthcare environment [7, 8]. Current measures to prevent the spread of the pathogen seek to block transmission routes and to limit inappropriate antibiotic usage [9]. The ability of spores to adhere to surfaces is thought to play an important role in their survival and spread and in the subsequent infection of susceptible individuals [9, 10]. Adherence to organic and inorganic surfaces is influenced by a number of factors, which include hydrophobicity and the presence of surface structures, such as appendages [11] and the outer spore layer known as the exosporium [12-15].

C. difficile shows significant variation within the species. There are over 100 existing ribotypes with each ribotype being a broad genetic group based on rRNA similarities [16]. Some ribotypes, such as the 027 ribotype are associated with higher virulence, and have been subject of study. In an earlier study we showed that clinical isolates of *C. difficile* varied widely in their inherent hydrophobicity and in their ability to adhere to stainless steel. These differences which were independent of ribotype, appeared to be linked to the presence of an exosporium-like layer [17]. Further characterisation of the spore morphotypes and particularly the exosporium layer was necessary to understand the variation both between different ribotypes and within the same ribotype, which became the focus of this research.

The exosporium is a loose outer layer, which surrounds the spores produced by some, but not all, *Bacillus* and *Clostridium* species, [12, 13, 18-20]. In the case of *Bacillus cereus* and *anthracis* species it forms a loose "baggy" layer surrounding the spore with hair-like appendages projecting from the surface [21]. Studies have reported that the outer spore layer of clinical isolates of *C. difficile* differ markedly [22]. For some isolates the exosporium appears to be tightly bound to the spore coat, which in others forms a bag-like structure [14, 22-24]. Several proteins were found to be essential for *C. difficile* spore outer layer assembly, including the *BclA*, *Cot* and *CdeC* families of proteins [25]. To add to our understanding of these proteins, we looked contribution of individual known spore structural proteins to spore surface morphology.

The analysis of spore surface structure is further complicated by the fact that there is no common spore production method making it difficult to compare the results between studies. To ensure that the results are relevant to the real-world properties of *C. difficile* virulence, it is important that spores grown in the lab are representative of the spores in the clinical environment. If the spore preparation method alters the outer spore layer, the spore properties could change significantly. For this reason,

we also sought to characterise the spore surface morphology depending on the method used to produce the spores to see if it had an effect on their appearance and surface properties.

2. Materials and Methods

2.1 Strains and growth conditions. The clinical isolates of *C. difficile* used in this study are shown in Table 1 and were obtained from the National Anaerobic Reference Unit, Cardiff, Wales. The CD630 strain and its mutants by insertional inactivation were obtained from the laboratory of Prof. Simon Cutting, University of Royal Holloway. The R20291, DS1748 and DS1813 originated from the National Anaerobic Reference Unit, Cardiff, Wales. Unless otherwise stated, all organisms were stored as spores at 4°C. Cultures were incubated at 37°C in a BugBox Plus anaerobic workstation (Ruskin Technology Ltd., Bridgend, United Kingdom) using an 85% nitrogen, 10% carbon dioxide, and 5% hydrogen gas mix.

2.2 Media composition

Wilson's broth [26] contained the following per litre: 90g Trypticase peptone, 5g Proteose peptone, 1 g Ammonium sulphate, 1.5 g Tris. The pH was adjusted to 7.4 following autoclaving. BHIS agar [24] contained the following: BHI agar + 5g/l yeast extract + 0.1% L-cysteine. For CD630 structural mutants, this agar was further supplemented with 5mg/ml erythromycin. Unless otherwise stated, all reagents were purchased from Sigma Aldrich, Dorset, UK. Unless otherwise stated, the centrifuge used in the purification steps was an Eppendorf 5417R centrifuge.

2.3 Spore production and purification methods

Lawley's method. The following method, based on the work of Lawley and colleagues [14], was employed to produce *C. difficile* spores. To produce spores, a single colony harvested from a BHI agar plate was used to inoculate 25ml of Wilson's broth, which was then incubated for 10 days at 37°C in anaerobic conditions.

To purify the spores, the cultured broth was centrifuge at 16,800 g for 15 min using a Beckman Coulter J-20 centrifuge, the supernatant was discarded, and the pellet was resuspended in 1.5ml distilled water. This washing step was repeated 4 more times using an Eppendorf 5417R centrifuge and the final pellet was resuspended in 1.5ml PBS. The spore suspension was then subjected to sonication for 90s using a tapered probe set at an amplitude of 35%, in a Soniprep 150 sonicator. Following sonication, the sample was mixed with 1.5ml of 10% Sarkosyl and incubated for 1 hour at 37°C with agitation. Samples were then pelleted at 3,400 g for 10 min and the pellets were resuspended in 1.5ml of PBS + 0.125 M Tris buffer (with pH 8) + 10mg/ml lysozyme and incubated overnight at 37°C with agitation. The suspensions were then sonicated again as described above, but with 1% Sarkosyl instead of 10% Sarkosyl prior to the 1-hour incubation.

The suspensions were then layered onto a 50% sucrose solution and centrifuged at 3,400 g for 20 min. The pellets were resuspended in 2ml of PBS containing 200 mM EDTA, 300ng/ml proteinase K + 1% Sarkosyl and incubated for 1 hour at 37°C with agitation. The suspensions were then layered on 50% sucrose and centrifuged as described above. The resulting pellets were washed with sterile distilled water (SDW) twice and finally resuspended in SDW and stored at 4°C.

Sorg's method. Described by Sorg & Sohenshein [6, 24], this method differs from that of Lawley in that the spores are produced on agar rather than in broth and the purification process is less

complex. Bacteria were incubated in BHIS agar anaerobically at 37°C for 4 days. Following incubation, cells were collected from the surface of the plate, using a 10µl inoculating loop and suspended in 1ml SDW in a sterile Eppendorf tube. The suspension was incubated at 4° C overnight and then centrifuged at 5000g for 5 min, the supernatant was discarded and the pellet was resuspended in 1ml ice cold water. This washing step was repeated 4 times. The suspensions were then layered onto a 50% sucrose solution and centrifuged at 3,400 g for 20 min and the pellet was resuspended in 1ml ice cold water. The suspension was then centrifuged at 5,000 g for 5 min, and the pellet was resuspended in 1ml ice cold water. This washing step was repeated 4 times. The resulting final pellet was resuspended in SDW and stored at 4°C.

Heeg's method. The following method is based on the work of Heeg and colleagues [23]. Bacteria were incubated on BHIS agar further supplemented with 250µg/ml cycloserine and 8µg/ml cefoxitin anaerobically at 37°C for 4 days. Following incubation, cells were collected from the surface of the plate, using a 10µl inoculating loop and suspended in 1 ml SDW in a sterile Eppendorf tube. The suspension was incubated at 4°C overnight and then centrifuged at 16,000 g for 4 min, with the supernatant and top layer of the pellet carefully removed after centrifugation. The rest of the pellet was resuspended in SDW and the washing step repeated 10 times. The resulting final pellet was resuspended in SDW and stored at 4°C.

Counting viable spores. To count the number of viable spores produced in all three of the above methods, a serial dilution and drop count was used. The spore suspension was serially diluted, mixing 10µl of the suspension with 10µl of SDW, with steps from 10^{-1} to 10^{-8} dilution. From each of the dilution steps, three 10µl drops were placed on a BHI agar plate supplemented with 0.1% sodium taurocholate (Joshi et al., 2012). Based on the number of colonies which were seen after a 48-h incubation, the original concentration of viable spores was calculated.

2.4 Visualisation of spores by light microscopy

A standard Gram staining method was used to stain the spores [27]. 10µl of spore suspension was placed on a glass slide and dried under a flame. The slide was subsequently flooded in crystal violet, Gram's iodine and safranin for up to 30 seconds in each step. Slides were washed with water following crystal violet and Gram's iodine flooding, and with ethanol following Safranin. Vegetative cells, if any, were stained and would appear as long purple rods. A Leica DM2500 microscope was used to visualise the spores, using x1000 magnification and oil immersion. Spores were then visualised using phase contrast settings in the microscope, with the slide under oil immersion.

2.5 Determining Spore Suspension Purity

Spore suspensions were gram-stained as described above. The ratio of vegetative cells (determined by gram staining) to spores (determined by phase contrast) was determined for 3 separate fields of view for each sample. The fields of view were chosen to contain at least 100 objects (spores or cells), but without clumping which could disrupt counts.

2.6 Visualisation of spores by Scanning Electron Microscopy (SEM)

A 10 µl aliquot of the purified spore suspension from stock was dried on a glass slide. The slide was then coated with a film of Gold-Palladium using the Agar Scientific Sputter Coater in three 15-second coating runs, using argon plasma. SEM images were captured using a Zeiss 1540 Crossbeam Scanning Electron Microscope using Inlens and SE2 imaging modes. Spore dimensions were

measured from SEM images using ImageJ software. To determine spore characteristics, 30 individual spores were used from at least 6 fields of view. The analysis of spore samples by electron microscopy was undertaken in the Cardiff School of Engineering.

2.7 Hydrophobicity assay

The Microbial Adhesion to Hydrocarbons (MATH) test was employed to determine the hydrophobicity of spores examined in this study [28]. A 3ml spore suspension in distilled water with an OD_{600} 0.4-0.6 was prepared in a McCartney bottle. OD measurements were made using an Ultrospec 1100 *pro* UV/Visible spectrophotometer, (Biochrom, Cambridgeshire, UK). A 300µl aliquot of hexadecane was then added to the suspension and vortex mixed (VortexGenie, Fisher Scientific, UK) for 1 min at room temperature. The mixture was incubated for 15 min at room temperature, allowing the layers to separate, with hexadane and hydrophobic components of the suspension rising to form to form the top layer, while the aqueous layer settled below with the hydrophilic components of the suspension. After this, the OD of the aqueous (bottom) layer was measured. The resulting decrease in the OD of the aqueous layer, compared to the the OD before the addition of hexadecane, was recorded.

2.8 Sonication

To determine the feasibility of removing the exosporium, we adapted the methods of Escobar-Cortes [29] and by Alyousef [30] which showed that intense sonication can remove the exosporium. For this investigation, 1ml spore suspensions with 10^7 spores each, were treated with 16 sonication cycles of sonication of 15µm amplitude for 50 sec each, and cooling on ice for 1 min between each cycle (Soniprep 150 sonicator, exponential probe).

2.9 Statistical tools

For simple statistical tools, including t-test and standard deviation, Microsoft Excel was used. For statistical tests where more than two data sets were compared, ANOVA tests were done using Graphpad Prism 5.0.

3. Results

3.1 Selection of the optimal growth and spore purification method

To support our studies to characterise differences in spore structure between different clinical isolates of *C. difficile* we first sought to identify a method which maximised spore yield and purity. As can be seen from Figure 1, both the Lawley and Sorg methods yielded similar levels of purified spores at 10^7–10^9 cfu/ml (per 25 ml of broth for Lawley's method and per agar plate with 25 ml of agar for Sorg's method) while the Heeg method only yielded 10^3 –10^4 cfu/ml per 25 ml agar plate.

To determine the reason for the low spore cfu when using the Heeg method, we compared spore numbers at different stages of the culture and purification process. Both the Lawley and Sorg methods produced spore counts ranging from 10^7–10^9 cfu/ml following primary culture and lost 47.5% and 40% of these spores, respectively, following purification. The final spore suspensions were of high purity, with more than 99% spores (Figure 2 A-B). In contrast, the Heeg method yielded only 10^4 cfu/ml upon primary culture of which 52.3% were lost during purification. The final suspension, when observed by light microscopy, was mostly composed of vegetative cells and debris

(Figure 2C). Overall, there was no statistically significant difference in percentage spores lost between the 3 methods (p=0.09). The results suggest that the poor spore yield using the Heeg method was due to a failure of the individual bacterial strains to produce spores in the cycloserine- and cefoxitin-enriched agar.

3.2 Physical dimensions of spores

The physical dimensions of the spores produced using the different production and purification methods varied markedly, ranging from 1.2 µm to 2.6 µm in length and from 0.7 µm to 1.15 µm in width (Figure 3). To determine if any of these results were significantly different, we compared the results obtained for each isolate produced using the Lawley and Sorg methods using Tukey's multiple comparison test. Overall, spores produced using the Lawley method were larger than the spores produced with the Sorg method. This difference was not significant for the strains DS1813 ($p > 0.05$) and CD630 but was significant for strains 1748 ($p = 0.011$ for length) and R20291 ($p<0.001$ for length).

To determine if the different spore production and purification methods had an effect on surface properties, spores produced by different clinical isolates of *C. difficile* were visualised by SEM. As can be seen from Figure 4, spores of different clinical isolates produced using the same method varied in their appearance. It is notable that despite belonging to the same "hypervirulent" 027 ribotype, the spore forms of DS1813 and R20291 differed markedly in appearance (Figure 4 C-F).

3.3 Effect of the spore purification method on spore structure

Next, we determined if the different spore production and purification methods had an effect on the overall appearance of spores of the same clinical isolate. While the different methods had no visible effect on the structure of spores produced by DS1813 this was not the case for R20291 spores. Spores of the R20291 strain purified using the Sorg method differed in structure from those purified using the Lawley method. The majority of spores (97%) purified using the Sorg method were surrounded by a loose layer. In contrast only 44% of the spores produced using the Lawley method were surrounded by a loose layer, likely due to the extra sonication and proteinase steps in the purification method. We also observed what appeared to be filaments, approx. 80 nm in width, extending from the spore surface (Figure 4F). The morphology of spores produced by DS1813 also varied depending on the production and purification method. 70% of the spores purified with the Sorg method had "ridge" structures as seen on Figure 4C. In spores purified with the Lawley method, only 21% showed these features (data not shown). For DS1748, the ratio of pineapple-like spores was higher when produced using the Sorg method (75% of those observed), and higher for DS1748 when produced using the Lawley method (99%). The ratio of spores with a bag-like layer was higher for both R20291 (98%) and CD630 (86%) when using the Sorg method.

To determine if spore coat protein mutants of CD630 differed in their appearance when produced using the two methods they were also subjected to SEM. However, we observed no obvious differences in the appearance (Figure 5) of the spore form of the various BclA and CotE mutants when compared to the parent strain.

3.4 Sonication of *C. difficile* strains

To obtain data on how different strains are changed by sonication and whether the sonication step in Lawley's method might have affected spore surface, the four strains purified with the Sorg method

were sonicated and then observed using SEM. Sonication resulted in the loss, to varying degrees, of the outermost surface features of the spores of all four clincial isolates (Figure 6). Sonication of the 630 and R20291 strains resulted in the removal of the loose layer which surrounds the untreated spore (Figure 4, blue arrows) in 49 and 85% of the total observed spores respectively. In the case of DS1813 the disruption was less marked with the loss of surface "ridge" stuctures from 39% of the spores while spores of DS1748 saw the least disruption with only 20.3% change (Figure 7). Sonication of DS1813, CD630 and R20291 yielded spores similar in appearance to those produced using the Lawley method. This was not the case for the 1748 strain, where the changed were stripped of the outermost layer, which could be observed separately from the spore (see example Figure 8).

3.5 Hydrophobicity of *C. difficile* spores

To determine the effect of the individual purification methods on spore properties, the commonly used Microbial Adhesion to Hydrocarbons (MATH) test was used. The relative hydrophobicities (RH) of the different strains are shown on Figure 7. Comparison of the RH values of the spores produced using the Lawley method revealed no statistically significant difference (ANOVA p=0.67), suggesting that all four strains possessed similar levels of hydrophobicity.

In contrast, when the spores form of the same clinical isolates were produced and purified using the Sorg method, we observed a statistically significant difference in RH values across all of the strains (ANOVA p=0.0001), with the spores of the DS1748 and DS1813 strains having the highest hydrophobicity and the R20291 strain having the lowest.

Sonication of the Sorg method-produced caused a significant reduction in RH values of all isolates when compared to the unsonicated Sorg spores ($p<0.0001$), suggesting that sonication had caused changes in spore surface properties. For the DS1813, CD630 and R20291, the sonicated strain also had lower RH values than the spores purified with Lawley method.

3.6 Structural mutant hydrophobicity

The hydrophobicity of the spores of the CotE and BclA insertional inactivation mutants was determined and compared to that of clinical isolates. Previous studies [32] have shown that BclA mutants have altered spore hydrophobicity, but the difference in methods made comparisons with isolates in our study not possible, so the hydrophobicity measurements were done with spores purified using the Sorg method.

As can be seen from in Figure 9, the knockout strains do show different hydrophobicities compared to the isogenic strain of CD630. The differences between CD630 strain mutants are significant overall (p=0.0004). The greatest differences were seen in the CotEn and BclA3 strains. CotEn is significantly higher in hydrophobicity than other strains (p=0.001). BclA3 was the lowest in hydrophobicity than other strains (p=0.006).

4. Discussion

Ideally a spore production method should minimize the damage to the final spores so that they mirror, as much as possible, the native form encountered in the context of disease. This is particularly important for a bacterium such as *C. difficile* which as a species exhibits markedly genomic plasticity due to horizontal gene transfer [33]. Thus, while isolates may share the same core genes and thus belong to the same genotype (depending on how it is defined) they may differ markedly in characteristics such as spore surface structure.

When we compared the spores produced by *C. difficile* belonging to the same hypervirulent ribotype (027) we observed marked difference in spore ultrastructure which was affected by the spore production method. R20291 spores produced two distinct morphotypes, smooth and surrounded by a bag-like layer. The relative proportion of each morphotype was influenced by the spore production method. While the majority of R20291 spores produced using the Sorg method were surrounded by a bag-like layer this number reduce to less than half using the Lawley method suggesting some form of physical disruption.

A similar change in the morphotype frequency was observed with DS1813 (Ribotype 027) and CD630 (Ribotype 012) suggesting that some aspect of the Lawley purification method may have an adverse effect on structural integrity. Unlike the Sorg method the Lawley purification protocol includes a sonication step. Independent sonication of the spores used in this study resulted in a higher proportion of smooth spores suggesting that this process may have contributed the shift in spore morphotype observed using the Lawley method. It must be noted that despite the sonication and loss out other layers feature, spores isolated using the Lawley method are slightly larger. The larger size of the spores produced during the Lawley method could be due to both differences in the media composition and due to the fact that it was a liquid culture, as opposed to growth on an agar.

Pineapple-like structures on the surface of *C. difficile* spores were reported previously [39], where it was thought to be a common feature among *C. difficile* spores in general. These features may also be related to the "bumps" observed using TEM on the surface of the spores of TL176, TL178 and R20291 strains of *C. difficile* which have been linked to the "thick" exosporium morphotype [35]. However, unlike Pizarro-Guajardo et al., we did not observe the presence of short hair-like structures surrounding our spores but did observe long hair-like features projecting from R20291 spores produced using the Lawley method. Overall, this illustrates how varied *C. difficile* spore morphology is varied between different clinical isolates and can present different phenotypes that can depend on the isolate and the methods used to culture and purify them. The individual results cannot be compares due to the major difference in methods of growth and isolation of *C. difficile* (summarised in Table 2).

As was the case for spore morphology, spore hydrophobicity also varied depending on the clinical isolates and the methods used to produce them. While spores from different isolates produced using the Sorg method varied markedly no such differences was seen when the spores were produced using the Lawley method. Sonicated of Sorg produced spores resulted in a decrease in the hydrophobicity of all of the isolates supporting the supposition that this step in the Lawley purification process could be responsible for these differences. This is line with previously reported results for the CD630 strains of *C. difficile* [29]. In this study we confirm this is also the case for

other strains of *C. difficile* and for "pineapple-like" spores the outermost layer can be observed separately from the rest of the spore. This opens up possibilities for further study of the composition and structure of this layer alone.

Why is this important? Studies have shown that *C. difficile* R20291 spores with a defective outer bag like adhere more efficiently to Caco-2 cells than their intact counterparts suggesting a potential role in adherence to epithelial surfaces and in the transmission of CDI [34]. Removal of the outer layer has also been shown to increase the ability of *C. difficile* spores to germinate [35]. If we are to fully understand the contribution of individual spore structures to the virulence of a particular isolate of *C. difficile* it is important, given the inherent diversity of the species, to employ methods which do not alter the structural integrity of the spores we are attempting to study.

For this reason, in this study we compared the appearance and hydrophobicity of spores produced using the Sorg method. The bag-like layer surrounding spores of R20291 and to a lesser extent CD630 were similar in appearance to that seen surrounding spores of the *Bacillus cereus* family [25]. In *B. cereus* the layer is hexagonal in structure around 8 nm in diameter and is composed of two proteins, *CotY* and *CotE* [21], with filament of a glycoprotein called *BclA* protecting from the surface [36].

The composition of the *C. difficile* outermost layer is not as well characterised as that of *B. cereus,* but in the case of R20291 this layer is based on two cysteine-rich proteins; *CdeC* and *CdeM* [37]. They are similar in nature to the *B. subtilis* proteins *CotY* and *CotZ.* As with *B. cereus,* the filaments projecting from the surface of *C. difficile* spores are composed of *BclA* homologs. In both *B. cereus and C. difficile* the filaments have been implicated in the attachment of the spore to bind to intestinal cells [38]. This attachment may be aided by the flexible nature of the bag-like layer which assists by maximising the contact area between the spore and cell surface.

The second major outer spore structure observed in this study, the "pineapple-like" layer, had previously been reported to be a common feature of *C. difficile* spore isolates [39]. In TEM studies this layer was observed to be electron dense and tightly bound to the inner spore. The structure of this layer to date has not been characterised.

It is possible that the "pineapple-like" structure may contribute to hydrophobicity, as they increase surface roughness and decrease wetting. Solid features of similar size to the "bumps" on the spore surface (125 nm diameter) are present on natural hydrophobic surfaces were they were shown to contribute to the hydrophobic properties [40].

In an attempt to determine the contribution of individual known spore structural proteins to spore surface morphology, we compared the appearance of wildtype CD630 spores to variants lacking individual structural spore proteins. Our failure to observe the structural deficiencies described in earlier work is probably a reflection of the fact that we employed SEM rather than TEM to visualise the spores [30, 31]. While SEM does not provide the level of magnification and resolution which can be achieved using TEM is does provide more information about the 3D shape of the structure which is extremely useful when attempting the characterise the surface architecture of spores.

In contrast to the data obtained from electron microscopy, our hydrophobicity results support the hypothesis [33] that the loss of the *C. difficile* homologs of BclA have an effect on surface chemistry and are similar to previously reported results. It is possible that the removal of this protein unmasked polar groups on the spore surface, without damaging the spore integrity. In contrast, the loss of the CotE homolog also failed to impact on hydrophobicity suggesting that the wild type protein is not surface exposed.

In conclusion, we have seen a variety we observed significant variation in the spore morphology of clinical isolates of *C. difficile*, due in part to the methods used to sporulate and purify them. Three distinct spore morphotypes were identified and the differences in these morphotypes were connected to different spore hydrophobicity. Sonication in particular can significantly change spore appearance and properties by removing the outermost layer of the spore. Finally, this work highlights the need for a common "standard" growth and purification method for *C. difficile* spores to allow for comparisons of results obtained by different research teams.

5. Acknowledgments

This work was funded by Cardiff University internal funding.

We would like to thank Dr. Emmanuel Brousseau from the Cardiff School of Engineering, Cardiff University for allowing the use of the electron microscopy facility and technical assistance with its operation.

We would also like to thank Prof. Simon Cutting, Royal Holloway, University of London for providing the *C. difficile* CD 630 knockout mutant strains.

6. References

1. Kelly CP, LaMont JT. 1998. Clostridium difficile infection. Annual review of medicine 49:375-390.
2. McNulty C, Logan M, Donald IP, Ennis D, Taylor D, Baldwin RN, Bannerjee M, Cartwright KA. 1997. Successful control of *Clostridium difficile* infection in an elderly care unit through use of a restrictive antibiotic policy. J Antimicrob Chemother 40:707-11.
3. CDC Website. http://www.cdc.gov/HAI/organisms/cdiff/Cdiff_infect.html Accessed 1/02/2019
4. UK Government. https://www.gov.uk/government/statistics/clostridium-difficile-infection-annual-data . Accessed 1/02/2019
5. Wiegand PN, Nathwani D, Wilcox MH, Stephens J, Shelbaya A, Haider S. 2012. Clinical and economic burden of *Clostridium difficile* infection in Europe: a systematic review of healthcare-facility-acquired infection. J Hosp Infect 81:1-14.
6. Sorg JA, Sonenshein AL. 2008. Bile Salts and Glycine as Cogerminants for *Clostridium difficile* Spores. Journal of Bacteriology 190:2505-2512.
7. Bartlett JG. 2006. Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. Ann Intern Med 145:758-64.
8. Best EL, Fawley WN, Parnell P, Wilcox MH. 2010. The potential for airborne dispersal of *Clostridium difficile* from symptomatic patients. Clin Infect Dis 50:1450-7.
9. Vonberg RP, Kuijper EJ, Wilcox MH, Barbut F, Tüll P, Gastmeier P, van den Broek PJ, Colville A, Coignard B, Daha T, Debast S, Duerden BI, van den Hof S, van der Kooi T, Maarleveld HJ, Nagy E, Notermans DW, O'Driscoll J, Patel B, Stone S, Wiuff C, Group ECd-IC, (ECDC) ECfDPaC. 2008. Infection control measures to limit the spread of *Clostridium difficile*. Clin Microbiol Infect 14 Suppl 5:2-20.
10. Kramer A, Schwebke I, Kampf G. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 6:130.
11. Rosenberg M, Doyle RJ. 1990. Microbial cell surface hydrophobicity. Am Soc Microbiol.
12. Faille C, Jullien C, Fontaine F, Bellon-Fontaine MN, Slomianny C, Benezech T. 2002. Adhesion of Bacillus spores and Escherichia coli cells to inert surfaces: role of surface hydrophobicity. Can J Microbiol 48:728-38.
13. Koshikawa T, Yamazaki M, Yoshimi M, Ogawa S, Yamada A, Watabe K, Torii M. 1989. Surface hydrophobicity of spores of Bacillus spp. J Gen Microbiol 135:2717-22.
14. Lawley TD, Croucher NJ, Yu L, Clare S, Sebaihia M, Goulding D, Pickard DJ, Parkhill J, Choudhary J, Dougan G. 2009. Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. J Bacteriol 191:5377-86.
15. Setlow P. 2003. Spore germination. Curr Opin Microbiol 6:550-6.
16. Stubbs, S. L. J., Brazier, J. S., O'Neill, G. L. & Duerden, B. I. (1999) PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. Journal of Clinical Microbiology, 37, 461.
17. Joshi LT, Phillips DS, Williams CF, Alyousef A, Baillie L. 2012. Contribution of spores to the ability of Clostridium difficile to adhere to surfaces. Appl Environ Microbiol 78:7671-9.
18. Charlton S, Moir AJ, Baillie L, Moir A. 1999. Characterization of the exosporium of Bacillus cereus. J Appl Microbiol 87:241-5.

19. Husmark U, Ronner U. 1992. The influence of hydrophobic, electrostatic and morphologic properties on the adhesion of *Bacillus* spores. 5:335-344.
20. Klavenes A, Stalheim T, Sjøvold O, Josefsen K, Granum PE. Attachment of *Bacillus Cereus* Spores With and Without Appendages to Stainless Steel Surfaces. Food and Bioproducts Processing 80:312-318.
21. Ball DA, Taylor R, Todd SJ, Redmond C, Couture-Tosi E, Sylvestre P, Moir A, Bullough PA. 2008. Structure of the exosporium and sublayers of spores of the *Bacillus cereus* family revealed by electron crystallography. Mol Microbiol 68:947-58.
22. Díaz-González F, Milano M, Olguin-Araneda V, Pizarro-Cerda J, Castro-Córdova P, Tzeng SC, Maier CS, Sarker MR, Paredes-Sabja D. 2015. Protein composition of the outermost exosporium-like layer of Clostridium difficile 630 spores. J Proteomics 123:1-13.
23. Heeg D, Burns DA, Cartman ST, Minton NP. 2012. Spores of *Clostridium difficile* clinical isolates display a diverse germination response to bile salts. PLoS One 7:e32381.
24. Sorg JA, Sonenshein AL. 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. J Bacteriol 192:4983-90.
25. Stewart, G. C. (2015). The exosporium layer of bacterial spores: a connection to the environment and the infected host. *Microbiology and Molecular Biology Reviews*, *79*(4), 437-457.
26. Wilson KH. 1983. Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. J Clin Microbiol 18:1017-9.
27. Beveridge TJ. 2001. Use of the gram stain in microbiology. Biotech Histochem 76:111-8.
28. Rosenberg M. 1991. Basic and applied aspects of microbial adhesion at the hydrocarbon:water interface. Crit Rev Microbiol 18:159-73.
29. Escobar-Cortés K, Barra-Carrasco J, Paredes-Sabja D. 2013. Proteases and sonication specifically remove the exosporium layer of spores of *Clostridium difficile* strain 630. J Microbiol Methods 93:25-31.
30. Alyousef, A. 2013. Identification and characterisation of lysin enzymes as potential therapeutics for the treatment of *Clostridium difficile*. PhD Thesis, Cardiff University.
31. Permpoonpattana P, Phetcharaburanin J, Mikelsone A, Dembek M, Tan S, Brisson MC, La Ragione R, Brisson AR, Fairweather N, Hong HA, Cutting SM. 2013. Functional characterization of *Clostridium difficile* spore coat proteins. J Bacteriol 195:1492-503.
32. Phetcharaburanin J, Hong HA, Colenutt C, Bianconi I, Sempere L, Permpoonpattana P, Smith K, Dembek M, Tan S, Brisson MC, Brisson AR, Fairweather NF, Cutting SM. 2014. The spore-associated protein BclA1 affects the susceptibility of animals to colonization and infection by *Clostridium difficile*. Mol Microbiol 92:1025-38.
33. Brouwer, M. S., Roberts, A. P., Hussain, H., Williams, R. J., Allan, E., & Mullany, P. (2013). Horizontal gene transfer converts non-toxigenic Clostridium difficile strains into toxin producers. *Nature communications*, *4*, 2601.
34. Paredes-Sabja, D., & Sarker, M. R. (2012). Adherence of Clostridium difficile spores to Caco-2 cells in culture. *Journal of medical microbiology*, *61*(9), 1208-1218.
35. Pizarro-Guajardo M, Calderón-Romero P, Castro-Córdova P, Mora-Uribe P, Paredes-Sabja D. 2016. Ultrastructural variability of the exosporium layer of *Clostridium difficile* spores. Appl Environ Microbiol 82:2202-9.

36. Kailas, L., Terry, C., Abbott, N., Taylor, R., Mullin, N., Tzokov, S. B., ... & Bullough, P. A. (2011). Surface architecture of endospores of the Bacillus cereus/anthracis/thuringiensis family at the subnanometer scale. *Proceedings of the National Academy of Sciences*, *108*(38), 16014-16019.
37. Barra-Carrasco, J., Olguín-Araneda, V., Plaza-Garrido, Á., Miranda-Cárdenas, C., Cofré-Araneda, G., Pizarro-Guajardo, M., & Paredes-Sabja, D. (2013). The *Clostridium difficile* exosporium cysteine (CdeC)-rich protein is required for exosporium morphogenesis and coat assembly. Journal of bacteriology, 195(17), 3863-3875.
38. Calderón-Romero, P., Castro-Córdova, P., Reyes-Ramírez, R., Milano-Céspedes, M., Guerrero-Araya, E., Pizarro-Guajardo, M., ... & Paredes-Sabja, D. (2018). Clostridium difficile exosporium cysteine-rich proteins are essential for the morphogenesis of the exosporium layer, spore resistance, and affect C. difficile pathogenesis. *PLoS pathogens*, *14*(8), e1007199.
39. Rabi, R., Turnbull, L., Whitchurch, C. B., Awad, M., & Lyras, D. (2017). Structural Characterization of Clostridium sordellii Spores of Diverse Human, Animal, and Environmental Origin and Comparison to Clostridium difficile Spores. *mSphere*, *2*(5), e00343-17.
40. Feng, L., Li, S., Li, Y., Li, H., Zhang, L., Zhai, J., & Zhu, D. (2002). Super-hydrophobic surfaces: from natural to artificial. *Advanced materials*, *14*(24), 1857-1860.
41. O'Connor, J. R., Lyras, D., Farrow, K. A., Adams, V., Powell, D. R., Hinds, J., ... & Rood, J. I. (2006). Construction and analysis of chromosomal Clostridium difficile mutants. *Molecular microbiology*, *61*(5), 1335-1351.
42. Carter, G. P., Lyras, D., Allen, D. L., Mackin, K. E., Howarth, P. M., O'connor, J. R., & Rood, J. I. (2007). Binary toxin production in Clostridium difficile is regulated by CdtR, a LytTR family response regulator. *Journal of bacteriology*, *189*(20), 7290-7301.
43. Girinathan, B. P., Monot, M., Boyle, D., McAllister, K. N., Sorg, J. A., Dupuy, B., & Govind, R. (2017). Effect of tcdR mutation on sporulation in the epidemic Clostridium difficile strain R20291. *Msphere,* 2(1), e00383-16.
44. Stabler, R. A., He, M., Dawson, L., Martin, M., Valiente, E., Corton, C., ... & Gerding, D. N. (2009). Comparative genome and phenotypic analysis of Clostridium difficile 027 strains provides insight into the evolution of a hypervirulent bacterium. *Genome biology*, *10*(9), R102.

8. Tables

Table 1. Strains of *C. difficile* used in this study.

Strain	Ribotype	Description
R20291	027	Hypervirulent ribotype, Stoke-Mandeville strain [17]
DS1813	027	Relatively high hydrophobicity of 87%, no visible exosporium [17]
DS1748	002	Relatively low hydrophobicity of 12% [17]
CD630	012	The first isolate of *C. difficile* to be genome sequenced [14]
CD630 BclA1^{-}	012	Deletion mutant of BclA homolog 1 [31]
CD630 BclA2^{-}	012	Deletion mutant of BclA homolog 2 [31]
CD630 BclA3^{-}	012	Deletion mutant of BclA homolog 3 [31]
CD630 CotE^{N-}	012	N-terminal deletion mutant of spore coat protein CotE [32]
CD630 CotE^{C-}	012	C-terminal deletion mutant of spore coat protein CotE. [32]

Table 2. Methods used in studies observing "pineapple-like" *C. difficile* Spores

Study	Imaging	Source of strains	Incubation media	Incubation Duration	Purification of Spores
Rabi et al., 2017	SEM and TEM	O'Connor et al., 2006 [41] Carter et al., 2007 [42]	Trypticase Yeast broth + with 0.1% sodium thioglycolate	10 days	Manual removal of top layer of pellet over multiple washes
Girinathan et al., 2017 [43]	TEM	Stabler et al., 2009 [44]	BHIS Agar	4 days	Density Gradient with 50% sucrose
Pizarro-Guajardo et al., 2016	TEM	Laboratory strains	3% Trypticase soy –0.5% yeast extract agar	5 days	Density Gradient with Nicodenz
This study	SEM	National Anaerobic Reference Unit, Cardiff, Wales	BHIS Agar	4 days	Density Gradient with 50% sucrose
	SEM		Wilson's Broth	10 days	Density Gradian + Sonication + Proteinase K

9. Figures and Legends

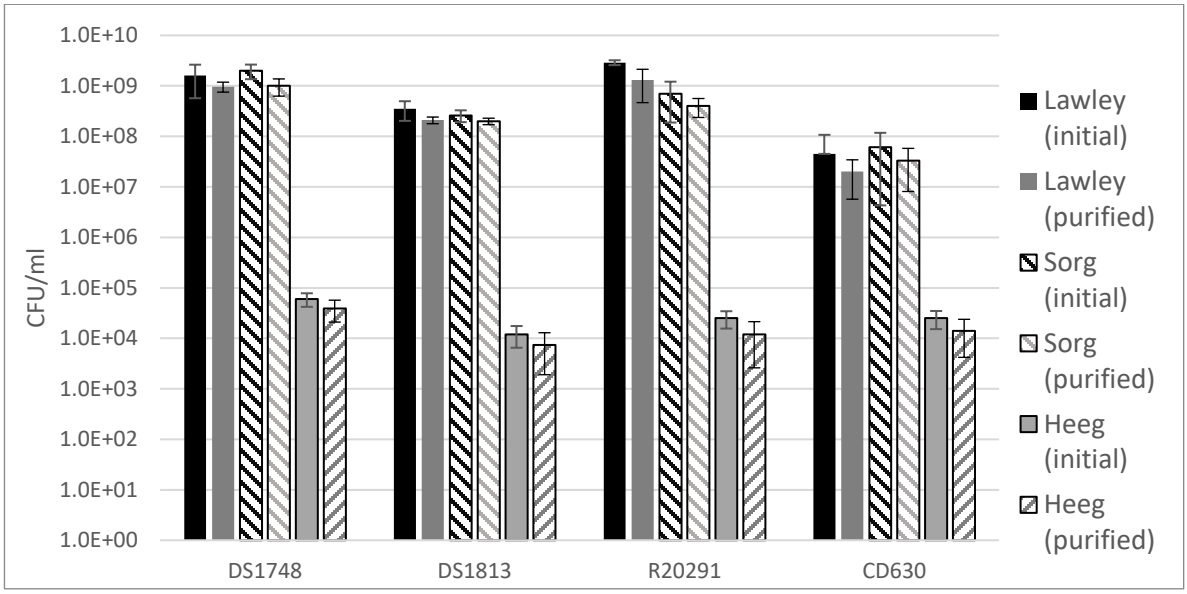


Figure 1. The initial yield of spores after growth in media and final yield of purified spores of clinical isolates of *C. difficile* obtained using three different production and purification methods (n=3).

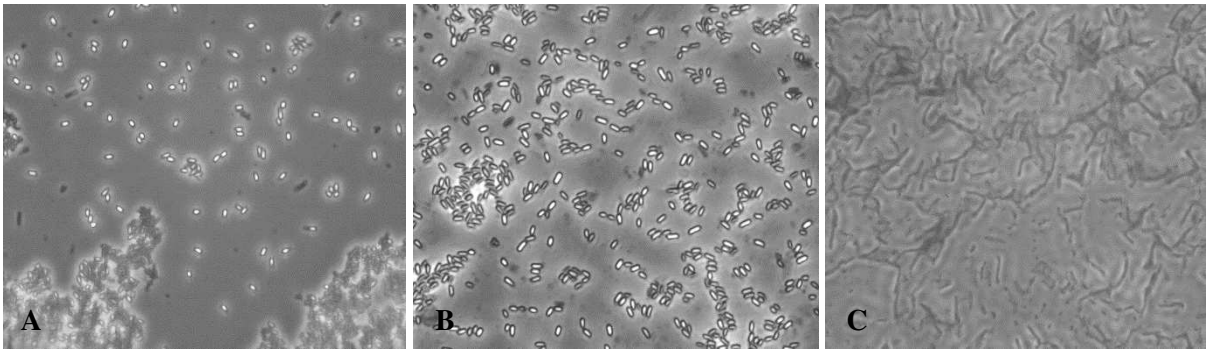
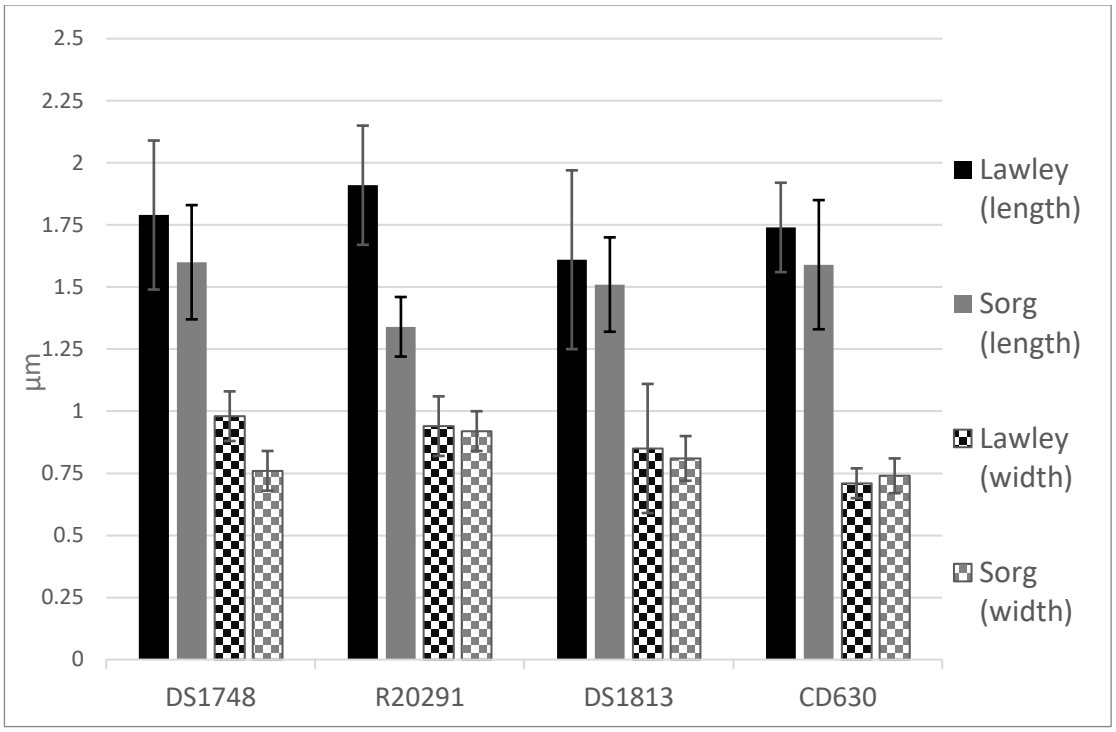


Figure 2. Phase contrast microscopy of *C. difficile* DS1748 spore suspension following purification using the Lawley method (A), Sorg method (B) and Heeg method (C). When purified with Lawley method or the Sorg method, the suspension is composed of mostly phase bright spores which can be clearly observed. However, with the Heeg method the suspension is mostly composed of vegetative cells and debris, while spores are not visible under phase contrast.



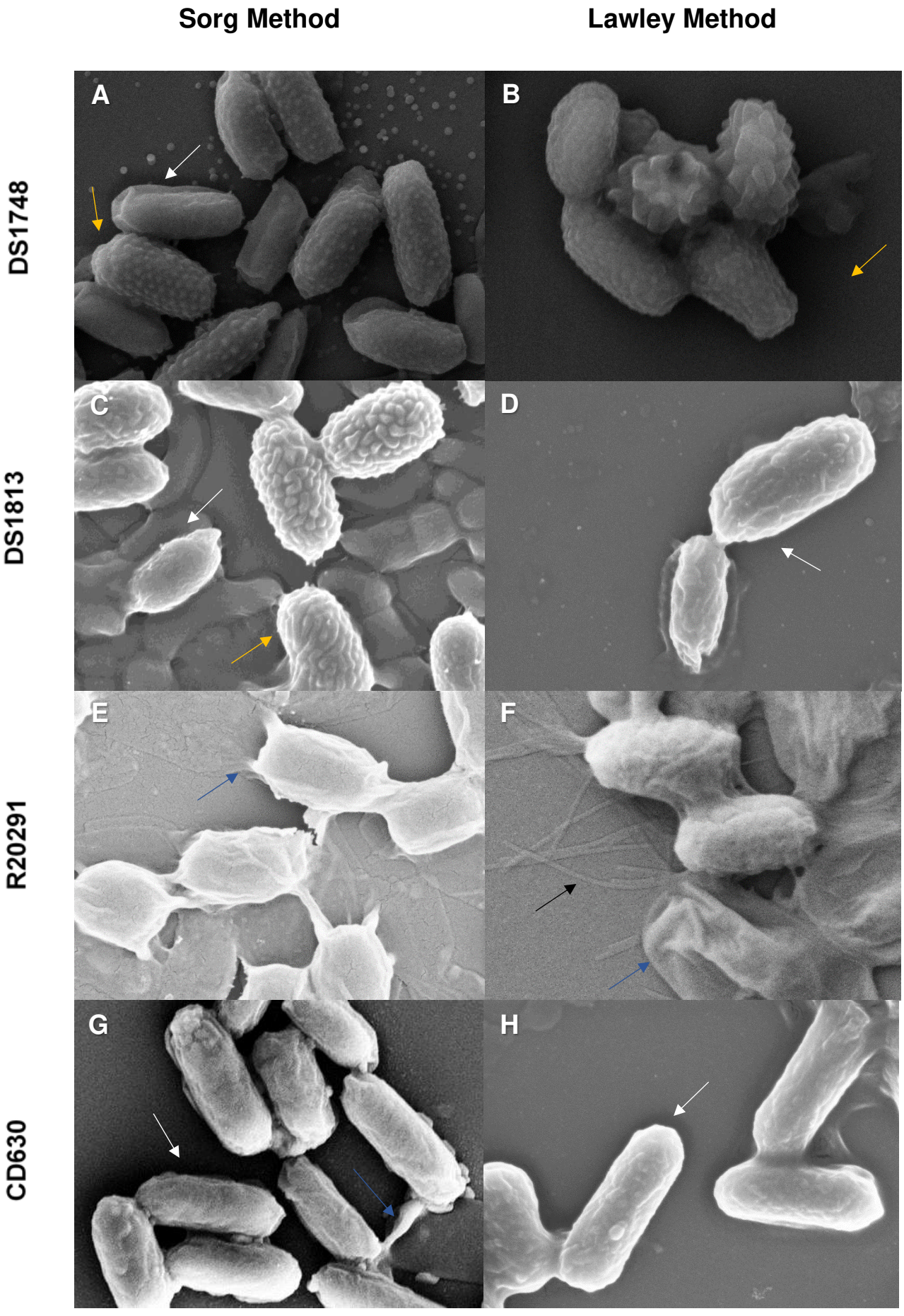
519

520 Figure 3. The effect of production and purification methods on the physical dimensions of spores of
521 four *C. difficile* isolates determined from SEM micrographs. 30 spores of each strain were measured.

522

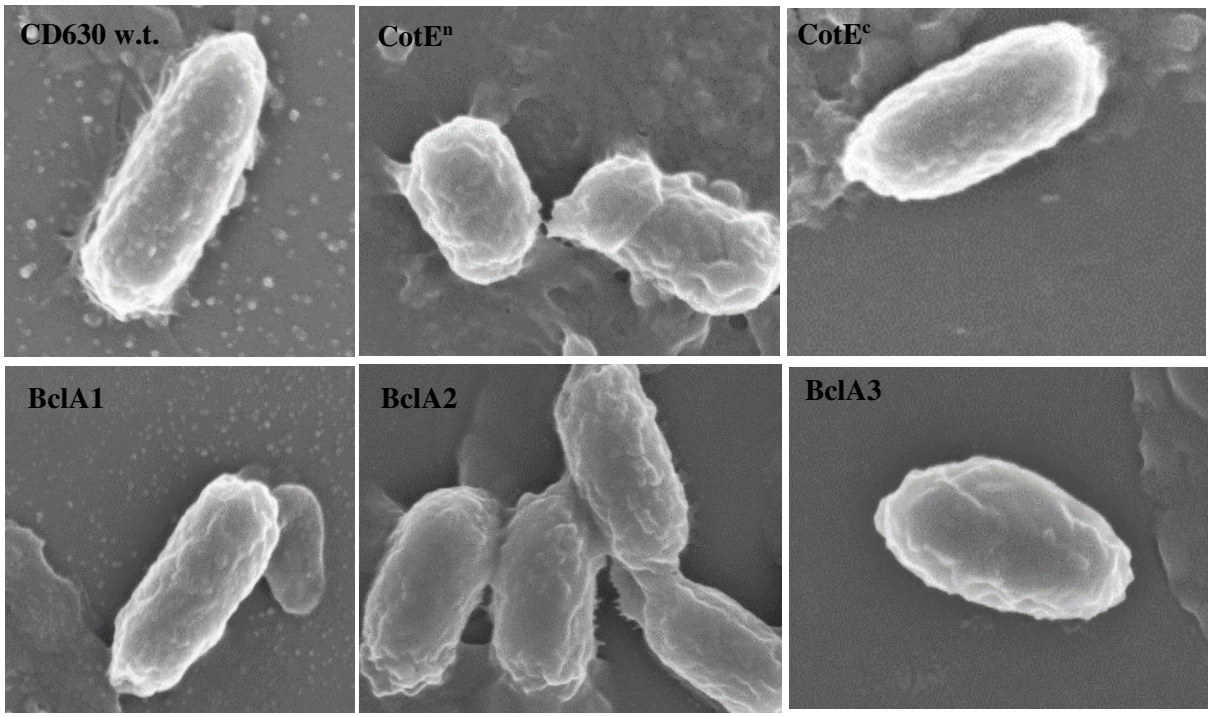
523

524


Sorg Method
Lawley Method
DS1748
DS1813
R20291
CD630
A
B
C
D
E
F
G
H

526 Figure 4. Surface morphotype variability of *C. difficile* depending on clinical isolate (rows) and
527 method of spore growth and purification (column). Arrows show examples of different morphotypes.
528 Orange arrows show examples of "pineapple-like" spores. Blue arrows show spores with an
529 associated with a bag-like layer. White arrows show plain smooth spores. Black arrows show
530 filaments extending from spores. Images are representative of a set of 30 fields of view for each
531 strain.

532



533

534 Figure 5. Spores of mutant strains of *C. difficile* CD630. No clear differences in surface feature was
535 seen. Images are representative of a set of 10 fields of view for each strain.

536

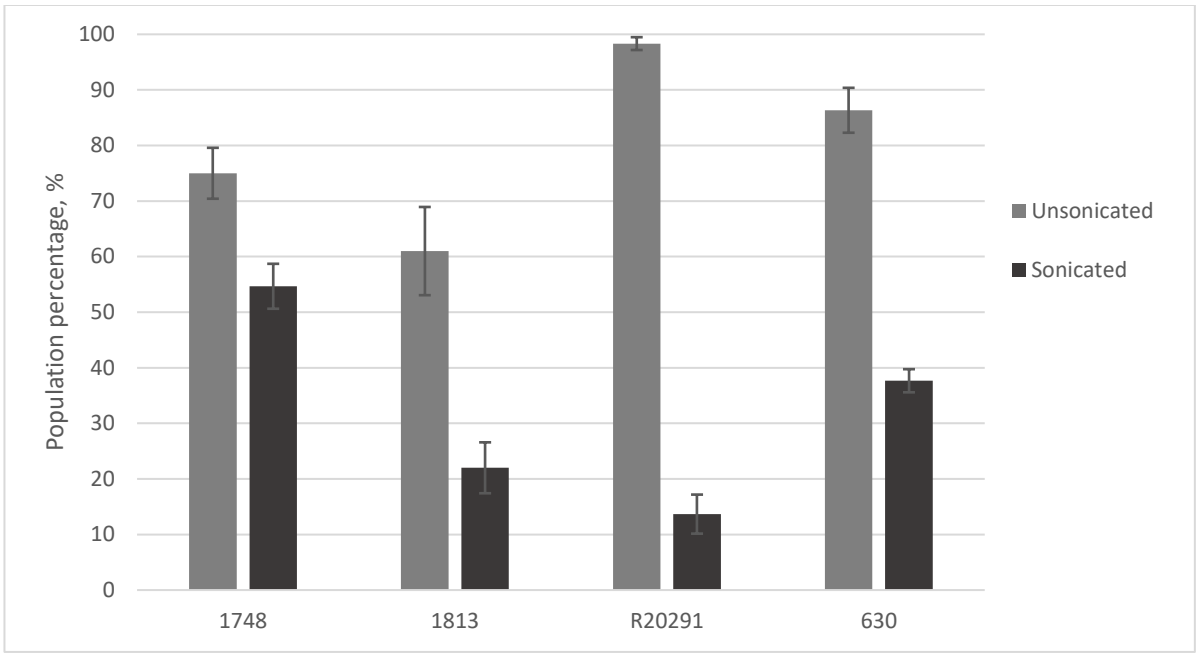


Figure. 6. Change in the relative proportion of spores with a recognisable feature (pineapple shape or loose exosporium) with and without intense sonication (n=3, 50 spores per repeat). The reduction is significant for all 4 strains ($p=0.005$ for 1748; $p=0.004$ for 1813; $p<0.001$ for R20291; $p<0.001$ for 630).

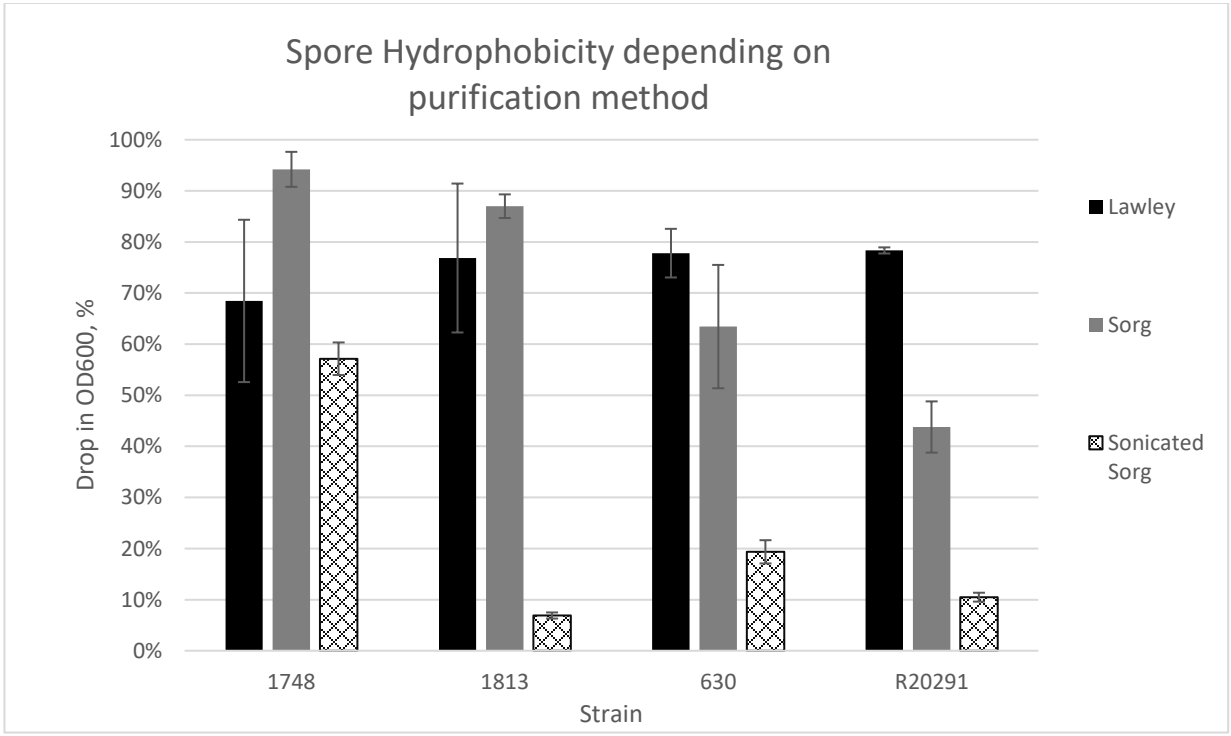


Figure 7. Relative hydrophobicities (RH) of spores of four clinical isolates *C. difficile* spores produced and purified using three different methods.

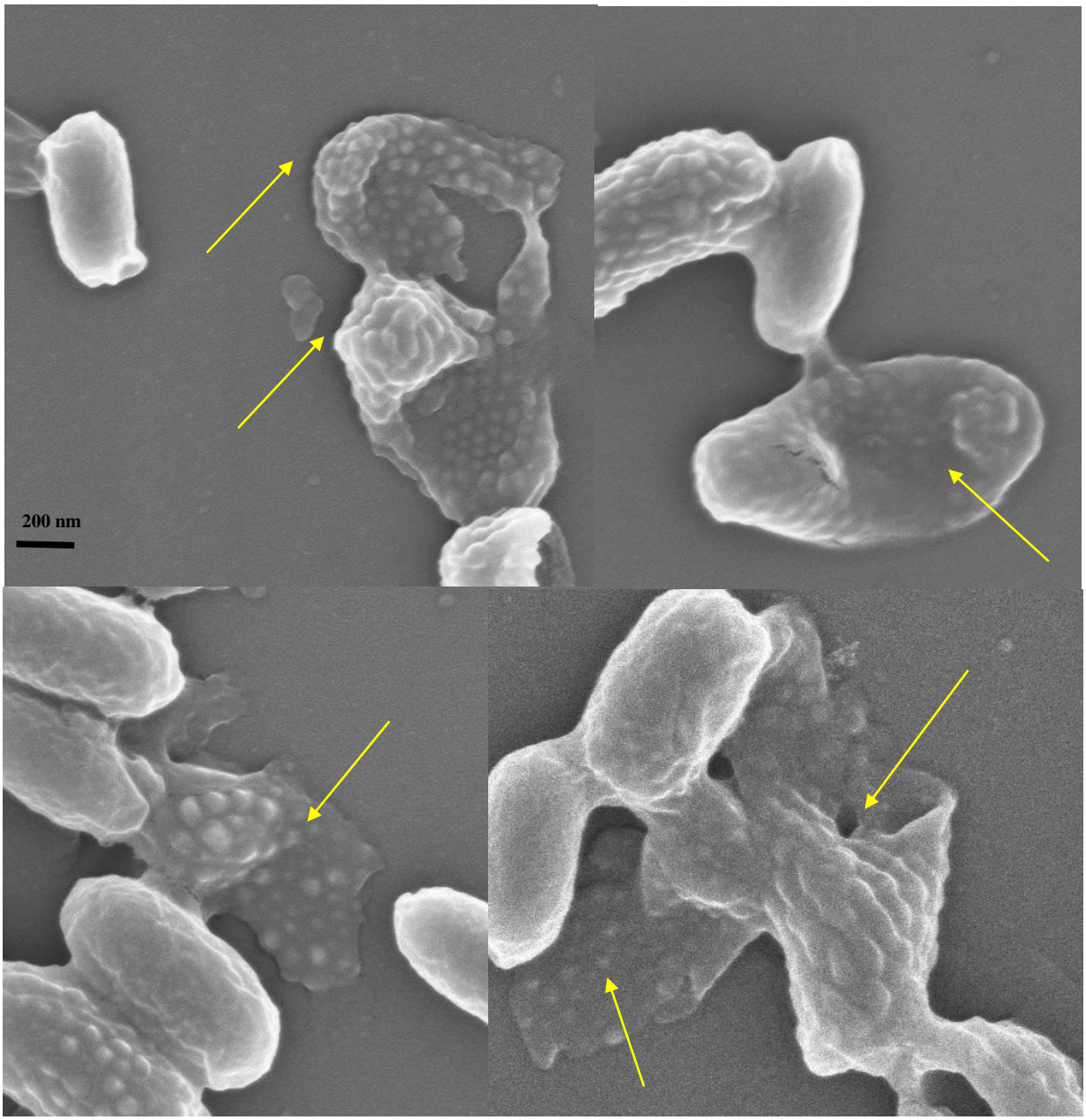
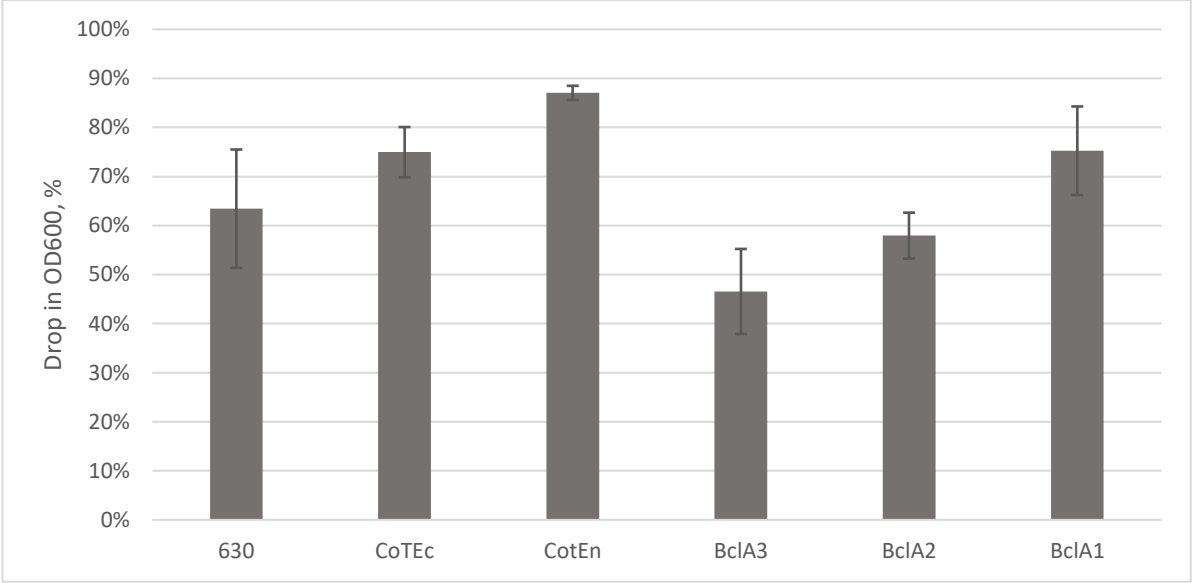


Figure. 8. Fragments of the outer “pineapple” layer of sonicated DS1748 spores are indicated by yellow arrows, and a “smooth” 1748 spore can see also be seen. These images are representative of 10 fields of view.



554

555 Figure. 9. The relative hydrophobicity of spores of *C. difficile* strain CD630 and its mutants deficient
556 in a structural protein. All spores produced and purified using the Sorg method (n=3 replicates of
557 each strain).

558